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Promoters regulated by the *glnG* (*ntrC*) and *nifA* gene products share a heptameric consensus sequence in the −15 region

(nitrogen regulation/positive activation)

DAVID W. OW*†, VENKATESAN SUNDARESAN*†, DAVID M. ROTHSTEIN‡§, SUSAN E. BROWN*†, AND FREDERICK M. AUSUBEL*†

*Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02138; and ‡Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT We have determined the nucleotide sequences of the *Klebsiella pneumoniae nifL* (regulation of N_2 fixation genes) and the *Escherichia coli glnA* (glutamine synthetase) promoters. We compared these sequences with the published sequences of three other promoters that, like the *nifL* and *glnA* promoters, are activated by the general nitrogen regulators *glnF* (*ntrA*) and *glnG* (*ntrC*). The three promoters are the *argTr* (arginine transport) and *dhuA* (histidine transport) promoters of *Salmonella typhimurium* and the *nifH* (nitrogenase) promoter of *Rhizobium meliloti*. All five sequences (with at most one mismatch) contain the heptameric consensus sequence T-T-T-T-G-C-A. In the *R. meliloti nifH* and *K. pneumoniae nifL* promoters, in which the transcription initiation sites have been determined, the consensus sequence is situated in the −15 region. We recently reported that the *K. pneumoniae nifA* product, which activates *nif* genes, can substitute for the *glnG* (*ntrC*) product in activating promoters of several genes involved in nitrogen assimilation, including the *nifL*, the *glnA*, and the *R. meliloti nifH* promoters. It is likely that *nifA* also activates the *S. typhimurium argTr* and *dhuA* promoters. In contrast, the *glnG* product cannot substitute for the *nifA* product in the activation of the *K. pneumoniae nifH* (nitrogenase) promoter. Consistent with this latter observation, and supporting the conclusion that the T-T-T-T-G-C-A sequence is a regulatory site for *glnG* product activation, the *K. pneumoniae nifH* promoter (C-C-C-T-G-C-A) has only partial similarity with the T-T-T-T-G-C-A consensus sequence in the −15 region.

Positive control of many nitrogen assimilation genes in enteric bacteria is mediated by the products of genes *glnF* (*ntrA*) (1–5) and *glnG* (*ntrC*) (3–7). Genes regulated in this manner include those governing the utilization of histidine (*hut*), proline (*put*), and arginine (*aut*), the transport of histidine (*dhuA*, *hisJQMP*) (8) and arginine (*argT*) (8), and anabolic genes involved in the assimilation of NH_4^+. The latter class is exemplified by the glutamine synthetase gene *glnA*, which has served as a model gene for the study of nitrogen regulation (for review, see ref. 9).

The enteric bacterium *Klebsiella pneumoniae* contains an additional nitrogen assimilation pathway not found in the other enteric species. This pathway is the enzymatic reduction of atmospheric dinitrogen (N_2) to ammonia (NH_4^+) (nitrogen fixation). A contiguous cluster of at least 17 *nif* genes arranged in seven or eight transcription units is involved in nitrogen fixation (for review, see refs. 10–12). Two of these genes, found in a single transcription unit (the *nifLA* operon), are responsible for regulation (12–17). The transcription of *nif* genes occurs only under conditions of nitrogen starvation and it has recently been shown that the *nifLA* operon acts as an intermediate in sensing this response (12, 16, 17). Specifically, activation of the *nifLA* operon is initiated under conditions of nitrogen deficiency by the general nitrogen control regulators, the products of *glnF* and *glnG*. Once transcription is initiated from the *nifLA* operon, the *nifA* product in conjunction with the *glnF* product activates all other *nif* promoters. It is believed that the *nifL* gene product remains inactive as a repressor unless a fixed source of nitrogen or oxygen is introduced into the system.

We have recently reported that the *nifA* product can substitute for the *glnG* product as a nitrogen control regulator, replacing the *glnG* product in the activation of its own promoter and, in addition, the promoters of several nitrogen assimilation genes, including the *hut*, *put*, *aut*, and *glnA* genes (12). This fact, along with other observations, led us to the proposal that *nifA* and *glnG* are evolutionarily related, and that *nifA* evolved directly from *glnG* in *K. pneumoniae* and thereby mediates stringent control of the nitrogen fixation pathway. Consequently, *nif* promoters (except for the *nifL* promoter), in contrast to the promoters of other nitrogen assimilation genes, cannot be activated by the *glnG* product. As a first step in elucidating the mechanism of *glnG*- and *nifA*-mediated gene activation, we have determined, and we present here, the DNA sequences of the *nifL* and *glnA* promoters, both of which are activated by the *glnG* or *nifA* product (12, 18). In addition, we report a comparison of the *nifL* and *glnA* promoters with four other *glnG*- or *nifA*-regulated promoters.

MATERIALS AND METHODS

Genetic Materials. *K. pneumoniae* strains: KP5617 is *hisD*$^-$ *hsdR*$^-$ *recA*$^-$ *srl*$^-$ *nifB*$^-$, KP5611 is *hisD*$^-$ *recA*$^-$ *srl*$^-$ *nifA*$^-$, and KP5614 is *hisD*$^-$ *hsdR*$^-$ *recA*$^-$ *srl*$^-$ (19). Plasmids: pGR116 carries the *K. pneumoniae nifLABQ* genes in pBR322 and has been described (20). pDO503 to pDO509 are described in the legend to Fig. 1. pgln8 is derived from pBR322 and contains a small part of the *Escherichia coli glnA* structural gene, including the 5′ upstream region, on a 1,200-base-pair (bp) *Eco*RI/*Cla* I fragment (18). pgln26, which contains a subregion of pgln8, is pBR322 carrying a 635-bp *Hae* III fragment (with *Hin*dIII linkers) that presumably contains sequences subject to regulation by the products of *glnG* and *nifA* (refs. 12 and 18; see text). pgln8 and pgln26 were gifts from K. Backman and Y. M. Chen.

Nucleic Acid Biochemistry. DNA sequences were determined according to the partial chemical degradation method of Maxam and Gilbert (21). S1 nuclease mapping was performed

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Abbreviations: bp, base pair(s); kb, kilobase pair(s).

† Present address: Dept. of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114.
§ Present address: Biogen, Inc., Cambridge, MA 02142.

as described by Berk and Sharp (22), using RNA that was synthesized *in vivo* and purified as described (23).

RESULTS

Structure of the *nifLA* Promoter. An 8.0-kilobase-pair (kb) *Eco*RI fragment in the *nif* cluster contains the two operons *nifLA* and *nifBQ* and a portion of the *nifF* gene (Fig. 1). Previous studies indicated that the unique endonuclease site *Bgl* II lies within the *nifL* structural gene and that the *nifL* promoter is probably located within 600 bp upstream (to the right in Fig. 1) of the *Bgl* II site (25). To delimit the *nifL* promoter, we defined its location functionally by using exonuclease *Bal* 31 to generate a set of deletions extending in both directions from the unique *Bgl* II site in pDO503 (see Fig. 1*a*). Deletion derivatives of pDO503 were recircularized with *Bam*HI molecular linkers and the extent of each deletion was determined by restriction endonuclease mapping analysis. The resulting set of *nifL* deletion plasmids was tested for complementation of a *nifA* mutation [complementation, which results in the ability to fix N_2 (Nif^+ phenotype), was not possible when this same *Eco*RI fragment was attached onto high-copy-number plasmid vectors due to inhibition of the chromosomal *nif* genes (20, 26) but was possible in this case because the vector used was the low-copy-number plasmid pRK248 (24)]. Because none of the deletions used for this analysis extended beyond the *Sal* I site in *nifL* and thus into the *nifA* gene, a Nif^- phenotype could be attributed to the deletion of regions governing *nifL* transcription or translation-initiation. (Alternatively, a Nif^- phenotype could also be due to polarity from fusion-generated stop codons.)

All 6 *nifL* deletion plasmids tested complemented the *nifB* mutation in KP5617, but only pDO504 and pDO505 complemented the *nifA* mutation in KP5611. Plasmids pDO506, pDO507, pDO508, and pDO509 have in common deletions extending beyond the *Xma* I (*Sma* I) site near the 5′ end of the *nifL* gene. This result suggests that the region upstream from the *Xma*I site contains the *nifL* promoter. To define the location of the *nifL* promoter precisely, we used the S1 nuclease mapping method (22). Total RNA extracted from derepressed cultures of *K. pneumoniae* was hybridized to purified coding strand of the *Xma* I/*Eco*RI fragment labeled with ^{32}P at the 5′ end of the *Xma* I site. Both the RNA extracted from KP5614 (wild type) and KP5614 harboring the multicopy plasmid pGR116 (which carries the *nifLA* operon) showed specific protection from nuclease S1 digestion of approximately 200 bp extending upstream from the *Xma* I end. This specifically protected length of DNA was observed only when RNA was extracted from cultures grown in the absence of NH_4^+ (data not shown; see ref. 27). This was consistent with the fact that plasmids pDO507, pDO508, and pDO509 (all of which have undergone deletions of this region) failed to complement the *nifA* mutation, indicating that this transcript comes specifically from the *nifLA* operon. In contrast, plasmid pDO506 contains the presumed transcription start point but nevertheless failed to complement. However, DNA sequence analysis of plasmid pDO506 revealed that it was missing the A-G-G-A-G ribosome binding site (28) (data not shown). This may result in premature termination of transcription.

We determined the DNA sequence of the region upstream from the *Xma* I site. The sequencing strategy consisted of end labeling the *Xma* I site of a purified *Xma* I/*Eco*RI fragment and the *Bam*HI sites present on deletion clones pDO506 and pDO507 (see Fig. 1*a*). The sequence was read upstream toward the *Eco*RI site until overlapping sequences were found. The sequences of both strands were determined for 420 bp upstream from the *Xma* I site. In Fig. 2, we show the DNA se-

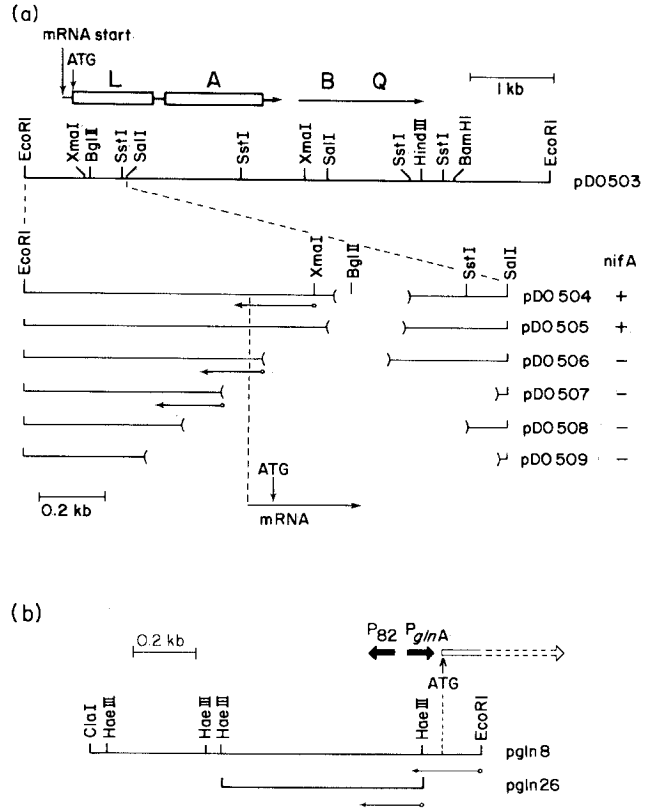

FIG. 1. (*a*) Physical and genetic maps of pDO503 and its *nifL* deletion derivatives (pDO504–pDO509). Construction of pDO503: The 8.0-kb *Eco*RI fragment (shown above) encodes the operons *nifLA* and *nifBQ* and is inserted into the *Eco*RI site of the low-copy-number plasmid vector pRK248 (24) in the orientation in which the tetracycline resistance (Tc^r) gene on the vector lies proximal to the *nifL* gene. The *Bgl* II site in the vector (not shown) and the nonessential *Bam*HI site on the *Eco*RI *nif* fragment were eliminated by filling the *Bgl* II (*Bam*HI) protruding ends followed by blunt end recircularization of the plasmid. These two modifications yielded plasmid pDO503, which contains a unique *Bgl* II site within gene *nifL* and lacks *Bam*HI sites. Deletion derivatives were generated *in vitro* by opening pDO503 with *Bgl* II, digesting the ends with exonuclease *Bal* 31, and recircularizing the plasmid in the presence of phage T4 DNA ligase and *Bam*HI molecular linkers. *Bam*HI linkers were used instead of *Bgl* II linkers because commercially available *Bgl* II linkers contain the T-G nucleotide sequence at their 3′ ends and the use of *Bgl* II linkers generates stop codons with an 8.3% probability. The extent of each deletion in derivatives pDO504 to pDO509 was determined by restriction enzyme analysis. Genetic complementation: Plasmids pDO504–pDO509 were tested for their ability to complement the *nifB* and *nifA* mutations in strains KP5617 (*nifB*$^-$) and KP5611 (*nifA*$^-$) with the acetylene reduction assay as a measure of successful complementation. All plasmids complemented the *nifB* mutation in KP5617. Derivatives pDO506 to pDO509 failed to complement the *nifA* mutation in KP5611. S1 nuclease mapping and DNA sequence data confirmed that pDO507–pDO509 are deleted for the *nifL* promoter, whereas pDO506 is deleted for the ribosome binding site. Structure of the *nifL* promoter: The strategy and extent of the DNA sequence determined is indicated by the arrows extending upstream (to the left in the figure) from the *Xma* I site of pDO504 and the deletion end points (*Bam*HI sites) of pDO506 and pDO507. The transcriptional and translational start sites were determined as described in the text. (*b*) Physical map of the *E. coli glnA* promoter region. Another promoter (P_{82}) present in this region and divergently transcribed from the *glnA* promoter had been identified by Backman *et al.* (18). The tentative locations of promoters for *glnA* (P_{glnA}) and the 82,000-dalton polypeptide gene (P_{82}) were previously assigned (18). The strategy and extent of the DNA sequence determination is indicated by the arrows extending upstream (to the left in the figure) from the *Eco*RI site of pgln8 and the *Hae* III site of pgln26. The translational start site of the *glnA* product was assigned as described in the text. Only endonuclease sites relevant to the text are shown.

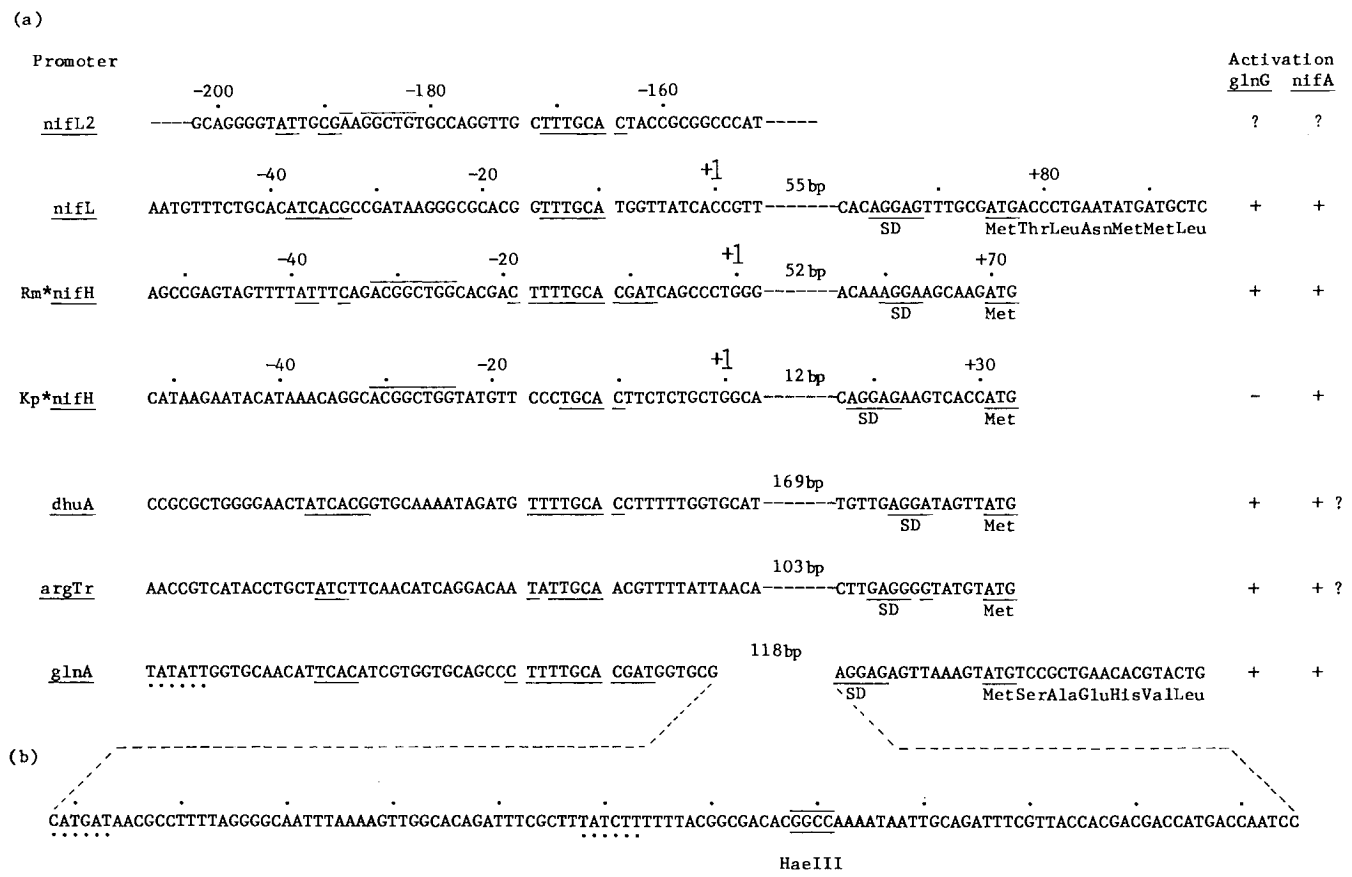

FIG. 2. Nucleotide sequence homology among promoters activated by *glnG*, *nifA*, or both. (*a*) The DNA sequences of the *nifL* and *glnA* promoters were determined by using the strategy outlined in the text and in Fig. 1. Only the promoter regions relevant to the text are shown in this figure. The sequences of the *nifH* promoters of *K. pneumoniae* (Kp*) and *Rhizobium meliloti* (Rm*) are from Sundaresan *et al.* (23). The sequences of the *dhuA* and *argTr* promoters were abstracted from Higgins and Ames (29). The *nifL2* sequence is included in this comparison because it shows homology to *glnG*- and *nifA*-activated promoters (see text). The transcriptional start sites of only the *nifL*, the *R. meliloti nifH*, and the *K. pneumoniae nifH* promoters have been determined (23, 27). Homologous sequences (mentioned in the text) are underlined or overscored. SD, Shine–Dalgarno sequence. (*b*) Nucleotide sequence of 118 bp of the *glnA* promoter region not included in *a. E. coli* consensus promoter-like −10 region sequences are underscored with dots. The *Hae* III site mentioned in the text is both underlined and overscored.

quence of the noncoding strand of regions of interest (see below).

When the nuclease S1-resistant (mRNA-protected) coding strand (labeled at the *Xma* I 5′ end) was subjected to electrophoresis next to a sequencing ladder (labeled at the same *Xma* I 5′ end), several mRNA start sites were found clustered at approximately 200 bp upstream from the *Xma* I site (data not shown; see ref. 27). The band with the strongest radioactive signal corresponded to the mRNA start point shown in Fig. 2. RNA extracted from cultures grown aerobically or at high temperatures (but in the absence of NH_4^+) also initiated transcription at this site (data not shown; see ref. 27).

At 64–68 bp downstream from the mRNA start site there is a presumptive Shine–Dalgarno (28) ribosome binding site followed closely by several possible ATG protein initiation codons. By aligning the DNA sequence with the NH_2-terminal amino acid sequence of the *nifL* protein [kindly provided by M. Chance and W. H. Orme-Johnson (personal communication)], we assigned the first ATG codon to code for the NH_2-terminal methionine of the *nifL* protein (Fig. 2). The −10 and −35 regions of the *nifL* promoter show poor homology to the Pribnow/Rosenberg and Court *E. coli* consensus promoter sequence (30, 31). This is consistent with the fact that the *nifL* promoter is inactive in the absence of positive control (12).

Structure of the *glnA* Promoter. Backman *et al.* have cloned and characterized the *E. coli glnA* gene (18). They identified the *glnA* promoter (see Fig. 1*b*) by showing that the insertion of a 635-bp *Hae* III fragment into the promoter probe vector λ132 (32) resulted in the regulated expression of β-galactosidase by the *glnG* product. We have recently reported that the expression of β-galactosidase from the cloned *glnA* promoter in λgln101 was also positively regulated by the *K. pneumoniae nifA* product (12), indicating that regulatory sequences recognized by *nifA* also reside on this 635-bp fragment. We determined the DNA sequence for about 200 bp on each side of the *Hae* III site at the "right" end of the 635-bp *Hae* III fragment as drawn in Fig. 1*b*. A DNA sequence beginning about 60 bp to the right of the *Hae* III site corresponds to the primary protein sequence reported for the first 21 amino acids of glutamine synthetase (33) [except for three differences: the previously reported amino acid sequence lacked methionine at the 1st position (Fig. 2), placed histidine rather than serine at the 4th position (Fig. 2), and lacked histidine at the 12th position between glutamate and glutamine (data not shown)].

About 12 bp upstream from the *glnA* ATG start codon is a Shine–Dalgarno sequence (28). Further upstream and within the *Hae* III fragment are three consensus promoter-like −10 region sequences (at −80, −130, and −180 bp from the ATG initiation codon, underscored with dots in Fig. 2) that are possible candidates for the *in vivo* promoter(s). Interestingly, the second upstream sequence is 10 bp downstream from a presumptive *glnG*/*nifA* regulatory sequence (see below). The DNA

sequence of the *glnA* promoter region relevant to this paper is shown in Fig. 2.

Comparison of Promoters Regulated by *glnG*, *nifA*, or Both. The DNA sequences of three promoters additional to the ones reported in this paper that require both *glnG* and *glnF* for activation have been reported. They are the *dhuA* (histidine transport) and *argTr* (arginine transport) promoters of *Salmonella typhimurium* (29), and the *nifH* (nitrogenase reductase) promoter of *R. meliloti* (23, 34). We have shown that the latter promoter, like the *glnA* and *nifL* promoters, also responds to *K. pneumoniae nifA*-mediated activation (23). It is likely that the *nifA* product can also activate the *dhuA* and *argTr* promoters because we have previously shown that the *nifA* product can substitute for the *glnG* product in the activation of the *aut* (arginine utilization), *hut* (histidine utilization), and *put* (proline utilization) genes in *E. coli* and *Klebsiella aerogenes* (12). The structure of the *K. pneumoniae nifH* promoter has also been reported recently (23). Functionally, it differs from the above promoters in that the *nifA* product, but not the *glnG* product, can activate its transcription (34).

Among the six promoters whose sequences have been determined, the mRNA transcriptional start sites have been determined for the *nifL*, the *R. meliloti nifH*, and the *K. pneumoniae nifH* promoters (23, 27). A comparison of the *nifL* and the *R. meliloti nifH* promoters reveals a common 6-bp sequence (T-T-T-G-C-A) in their respective −15 regions (Fig. 2). The probability of encountering the same 6-bp sequence randomly is about 1 in 4,000. Similar sequences were found in the *glnA*, *dhuA*, and *argTr* promoters (see Fig. 2), about 130–190 bp away from the ATG translational start codons. The sequence T-T-T-T-G-C-A appears to be a consensus sequence for all of these promoter regions (except the *K. pneumoniae nifH* promoter) because each of the five promoters contains at least 6 out of the 7 bp of T-T-T-T-G-C-A. In addition, the *dhuA* and the *R. meliloti nifH* sequences show 8 bp (T-T-T-T-G-C-A-C) in common. Most striking is the 12-bp sequence (C-T-T-T-T-G-C-A-C-G-A-T) shared between the *R. meliloti nifH* promoter and the *glnA* promoter region. In the case of the *nifL* and the *R. meliloti nifH* promoters, it is known from S1 nuclease mapping experiments that this consensus sequence lies within a critical region for regulation. In the case of *glnA*, *argTr*, and *dhuA*, however, it is not known whether the consensus sequence is actually within the promoter. We cannot rule out the possibility that the sequence merely reflects a fortuitous evolutionary similarity.

The −35 regions of the *K. pneumoniae nifL* and the *R. meliloti nifH* promoters give only weak evidence of homology. When the −35 regions of these promoters were compared to the corresponding regions (upstream from the presumptive consensus T-T-T-T-G-C-A sequence) of the *dhuA*, *argTr*, and *glnA* promoters, homology was observed only between the *nifL* and *dhuA* sequences. These promoters share the 6-bp sequence A-T-C-A-C-G.

Interestingly, we found a stretch of sequence about 150 bp upstream from the *K. pneumoniae nifL* promoter that is very similar to the *R. meliloti nifH* promoter sequence. This presumptive second *nifL* ("*nifL2*" in Fig. 2) promoter may respond to *glnG*- or *nifA*-mediated activation, although transcripts originating from this region were not observed by using the S1 nuclease mapping technique described above, at least not under conditions in which transcription was activated by the *glnG* product.

Among the six promoters compared in Fig. 2, only the *K. pneumoniae nifH* promoter fails to respond to the *glnG* product. The −15 region of this promoter, which responds only to the *nifA* product, does not contain T-T-T-T-G-C-A but rather C-C-C-T-G-C-A. Because all six promoters probably respond to the *nifA* product, it is possible that the sequence T-G-C-A, common to all six promoter regions, is necessary for *nifA*-mediated gene activation, although this sequence alone is most likely insufficient. This conclusion is based on the argument that the probability of encountering a 4-bp sequence is relatively high. A more plausible explanation would be that *nifA*-mediated transcription requires either the presumptive *glnG*/*nifA* recognition sequence T-T-T-T-G-C-A or T-G-C-A in conjunction with another 8-bp sequence, A-C-G-G-C-T-G-G (Fig. 2, overscored), that is found in the −30 region of both the *K. pneumoniae* and *R. meliloti nifH* promoters (23).

DISCUSSION

Transcriptional start points for the *dhuA* and *argTr* promoters that lie 120 and 60 bp, respectively, downstream from the T-T-T-T-G-C-A sequence have been suggested previously. In the case of the *argTr* promoter, Higgins and Ames (29) assigned the −10 region on the basis of an *E. coli* consensus promoter-like sequence. Preliminary results indicate that *in vivo* transcription does start near this previously proposed promoter (G. F.-L. Ames, personal communication). In the case of the *dhuA* promoter, the −10 region was assigned on the basis of both an *E. coli* consensus promoter-like sequence and on a promoter-up point mutation (29). Consensus promoter-like sequences can also be found at 10 and 65 bp downstream from the T-T-T-T-G-C-A region of the *glnA* promoter to the left of the *Hae* III site (Fig. 2*b*, underscored with dots).

Consistent with the possibility that transcription of these three promoters does not start near the T-T-T-T-G-C-A sequence is the fact that the T-T-T-T-G-C-A sequence in each promoter is quite far from the ATG translation initiation codons (see Fig. 2). On the other hand, consensus promoter-like sequences are located at approximately 75, 60, and 60 bp from the ATG translation initiation codons of the *argTr*, *dhuA*, and *glnA* promoters, respectively. The T-T-T-T-G-C-A sequences in the *nifL* and *R. meliloti nifH* promoters are only about 80 bp away from the ATG start codons. In the following paragraph, we propose a model for the transcriptional control of the five promoters discussed above that takes into account the apparent differences in the structures of the *nifL* and *R. meliloti nifH* promoters on the one hand and the *glnA*, *dhuA*, and *argTr* promoters on the other.

The *nifL* and *R. meliloti nifH* promoters share the feature that both promoters exhibit an absolute requirement for *glnG*- or *nifA*-mediated activation. In contrast, the *glnA*, *dhuA*, and *argTr* promoters constitute a second group of *glnG*/*nifA*-regulated promoters because they are transcriptionally active at a basal (lower) level even when cells are grown in excess NH_4^+ and glutamine, suggesting that positive regulation is not required for basal activity. It is possible that the basal level of transcription of the latter three promoters is due to initiation at the poor but recognizable consensus promoter-like sequences and that the *glnG*/*glnF*- or *nifA*/*glnF*-induced levels of transcription are due to initiation near the T-T-T-T-G-C-A sequences. Alternatively, under activating conditions, transcription may still be initiated at the presumptive consensus promoter-like sequence. However, recognition of the T-T-T-T-G-C-A sequence by *glnG* (or *nifA*) and *glnF* products may enhance transcriptional efficiency at the downstream transcriptional start site. For example, transcriptional factors could be shuttled downstream from the T-T-T-T-G-C-A site or transcriptional factors binding at the T-T-T-T-G-C-A sequence could promote cooperative binding of transcriptional factors at the downstream site.

The latter hypothesis is interesting in light of the second T-T-T-T-G-C-A sequence in the *K. pneumoniae nifL* promoter at −170 bp upstream from the transcriptional start site. Because this region of the *nifL* promoter shows remarkable similarities to the *R. meliloti nifH* promoter, which is activated by *nifA* product, it is possible that auto-activation of the *nifLA* operon by the *nifA* product occurs at this upstream sequence. On the other hand, mRNA transcripts have not been mapped to this region. An alternative explanation for a function for this region may be analogous to that described for the *glnA*, *dhuA*, and *argTr* promoters. That is, the *nifL2* region may function as an initial binding site to shuttle transcriptional factors to the downstream promoter, or the binding of transcriptional factors to the *nifL2* locus may promote cooperative binding at the downstream promoter.

Finally, it is interesting that the sequence homology that exists among the *K. pneumoniae nifL*, the *R. meliloti nifH*, and the *K. pneumoniae nifH* promoters occurs at the −15 and −30 regions. Prokaryotic RNA polymerases are believed to recognize promoters at the −10 and −35 regions (30, 31, 35). This suggests that the *nifA* and *glnF* or *glnG* and *glnF* products may be modifying RNA polymerase to affect its ability to recognize these particular −10 and −35 sequences.

In closing, we would like to note that Drummond *et al.* have independently determined the nucleotide sequence and the start point transcription of the *nifL* promoter (36), which are in complete agreement with the results presented here. A preliminary report of this work was presented at the North Atlantic Treaty Organization symposium in Porto Portese, Italy, August 1982.

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